

Determination of the Levels of Aflatoxin in Peanut Butter using the Aflaprep Immunoaffinity Column Clean-Up Procedure: Collaborative Trial

Fiona M. Worner, Alan L. Patey and Roger Wood

Food Science Laboratory, Ministry of Agriculture, Fisheries and Food, Colney Lane, Norwich, NR4 7UQ, U.K.

Summary:

Twenty one United Kingdom laboratories participated in a collaborative trial to evaluate the Aflaprep immunoaffinity column for the determination of aflatoxins in peanut butter. Each participant received ten randomly numbered samples of roasted peanut butter which were prepared as five sets of blind duplicates. One set of samples was blank, uncontaminated peanut butter. The other four sets of samples contained different concentrations of total aflatoxin - ca 8; 12; 35; 50 µg/kg. These latter samples were prepared from naturally contaminated peanuts, the major contamination being from aflatoxin G₁. Participating laboratories were instructed to follow a set procedure with regard to the use of the immunoaffinity column for extraction and were to quantitate total aflatoxin levels by HPLC with post-column derivitisation with iodine. The relative standard deviations for reproducibility (RSD_R) for the total aflatoxin levels, from the twenty one laboratories, for the four concentrations of contaminated peanut butter, were respectively; 54% (on a mean of 7.0 µg/kg); 30% (on a mean of 11.3 µg/kg); 35% (on a mean of 33.2 µg/kg); and 36% (on a mean of 47.7 µg/kg).

Introduction:

The four aflatoxins B₁, B₂, G₁, and G₂ are toxic metabolites of the fungi *Aspergillus flavus* and *A. parasiticus*^{1,2}. They are potent liver toxins showing carcinogenic properties, with aflatoxin B₁ being the most toxic compound. They can contaminate some cereal crops and ground nuts and their products.

One of the problems concerning the determination of aflatoxins in foodstuffs has been the sample extraction procedure. The immunochemical analytical methods which have been devised simplify and shorten the time required for the clean-up of extracts of aflatoxin from foodstuffs^{3,4}. The immunoaffinity column contains a gelatinous suspension of monoclonal antibodies covalently attached to a solid support. Any aflatoxin present in the sample is retained by the antibody within the gel suspension. The column is washed to remove any extraneous non-specific material. The eluate is collected by elution of a suitable organic solvent for analysis by either high performance liquid chromatography (HPLC) or thin layer chromatography (TLC).

Two previous collaborative trials^{5,6} evaluated an immunoaffinity column clean-up procedure to determine the aflatoxin levels in peanut butter. Participants in these trials were expert laboratories routinely carrying out aflatoxin analyses. The specific column used was the Total Easi-Extract column from Biocode, UK. In the first trial⁵, which involved 10 UK laboratories, each

laboratory had to follow a protocol for the use of the column but could choose their own end detection method. In that trial the two levels of aflatoxin B₁ contamination were 12 and 35 µg/kg respectively. Relative standard deviations for reproducibility⁷ (RSD_R) for the two levels were 46% and 36%. The second collaborative trial⁶ involved 13 laboratories from 7 different countries and analysed total aflatoxin concentrations present. Another difference between this and the first trial was that the instrumental method used was strictly specified namely HPLC with post column derivitisation with iodine. The mean levels of total aflatoxin found were 4, 15, and 35 µg/kg with RSD_R values between 33 and 45%. It was concluded that more development was needed before this particular method could be submitted for adoption as an AOAC approved method because the RSD_R values were too high. This report describes a study of twenty one UK laboratories (mostly Public Analysts) using the May & Baker Aflaprep immunoaffinity column for the preparation of peanut butter extracts for aflatoxin analysis. The end-point determination was by HPLC, but the method to be used (see Appendix I, Section 5.8) was not as closely defined as in the second Biocode trial.

The trial was carried out on samples of peanut butter which contained concentrations of total aflatoxins close to 10 µg/kg and at levels significantly above that value. 10 µg/kg is the most common statutory limit proposed or introduced by regulatory authorities for aflatoxin contamination of nuts and nut products⁸.

Organisation of the Trial:

The trial was organised by the Food Science Laboratory, MAFF, in conjunction with BFMIRA who supplied the samples and May & Baker Diagnostic Limited who provided immunoaffinity columns and the protocol for the extraction and HPLC determination.

Participants:

Nineteen public analysts, one food manufacturer's laboratory and the Laboratory of Government Chemist participated in the trial.

Method, Reagents, Apparatus and Procedure Used:

The method used in this trial is given in Appendix I. It requires the quantitative determination by HPLC of the individual aflatoxins present in peanut butter after using the Aflaprep Immunoaffinity Chromatography for sample clean-up.

Sample Preparation:

The samples were prepared by BFMIRA U.K. who were asked to prepare a "blank" and four contaminated butters at levels between 5 - 60 µg/kg total aflatoxins. The contaminated butters were prepared by blending high level contaminated butter with uncontaminated butter. The highly contaminated butter was produced from naturally contaminated peanuts. Homogeneity data for each of the samples were considered to be satisfactory (Coefficient of variation for total aflatoxins > 8%, n=10 for each sample).

For the four samples the percentage of each aflatoxin present (as a function of the total) was the same. About 70% of the total aflatoxin contamination was caused by G₁, about 13% of the aflatoxin contamination was caused by B₁, with G₂ and B₂ being responsible for 5% and 12% contamination respectively. The distribution of aflatoxins in the trial samples is unusual even though prepared as

a result of natural contamination. Normally B_1 would be the predominant aflatoxin rather than G_1 . Nevertheless it was concluded that using these samples would provide an additional evaluation of the method. Each participant was given 10 samples (5 pairs, blind duplicates) each coded with an individual 3 figure number.

Results:

Participants were asked to report their results for all four levels of aflatoxins obtained, uncorrected for recovery, as this is the preferred approach in submitting data for collaborative trials⁷. The results received from each participant are shown in tables I - V. Each table gives the concentration of aflatoxins B_1 and G_1 present in each of the blind duplicates. Each table also shows the total aflatoxin concentration ($B_1 + B_2 + G_1 + G_2$) present in each sample. The individual values of B_2 and G_2 were very small in comparison with the individual values of B_1 and G_1 (being of the order of about 10%) and so were only considered as part of the total aflatoxin concentration.

Statistical Analysis of the Results:

The results obtained were statistically analysed as outlined in the AOAC/ISO/IUPAC harmonised protocol⁷ for the analysis of collaborative trial data. For the detection of the outliers two statistical tests are used. Cochran's maximum variance test ($P < 0.05$) was used to detect the presence of laboratories with an exceptionally high value for the range. Grubbs test ($P < 0.05$) was used to determine whether the extreme values (highest and lowest) for the laboratory means are sufficiently different to warrant their removal from the data set. Outlying results are marked in the tables of results.

For tables I - V, mean concentrations for the samples containing different levels of aflatoxin were calculated on results remaining after removal of outliers. The repeatability (r), reproducibility (R), their standard deviations (SD_r , SD_R), and the repeatability and reproducibility relative standard deviation (RSD_r , RSD_R) were also calculated for the naturally contaminated peanut butters; these values are given in tables II - V.

Discussion:

The peanut butters analysed consisted of a blank and four contaminated samples. The level of aflatoxin (consisting entirely of aflatoxin B_1) in the blank was very low (total 0.19 $\mu\text{g/kg}$) with many of the results being reported as below the participants' limits of detection. All these results have been noted in table I as 0.0 and because of the large number of such results it was decided only to obtain a mean value for these data, after removal of outliers, as any other more complex statistical evaluations would not produce meaningful results. For the other four samples, where a more detailed statistical evaluation of the results was carried out, mean levels for total aflatoxin were found to be 7.0, 11.3, 33.2 and 47.7 $\mu\text{g/kg}$. The mean levels of the first two of these samples straddle the common 10 $\mu\text{g/kg}$ statutory limit⁸. RSD_R values were 54% and 30% respectively. This latter figure is probably not significantly better than the RSD_R values for the two high level peanut butters which were 35 and 36%. RSD_R of the assay would not be expected to be greater at 11 $\mu\text{g/kg}$ than at 48 $\mu\text{g/kg}$.

In general a collaborative trial is aimed at evaluating the analytical procedure without specifying or endorsing a particular product. However when immunoaffinity columns are being used the performance of the method is dependant on, and thus linked to, the specific column being tested. In table VI

the RSD_R values, for the total aflatoxin levels in the samples, are compared with those from previous trials using the Biocode immunoaffinity column. The RSD_R values for the total aflatoxin levels, for the Aflaprep column, ranged from 30-54%. These were compared with the RSD_R values from the Biocode column trials, which were 33-46%. All these trials analysed samples containing a similar range of aflatoxin contamination. From these results it can be concluded that there is no significant difference in RSD_R values for each of the columns that have been subject to collaborative tests for the determination of aflatoxin levels in peanut butter.

The samples which were analysed showed an unusual pattern of aflatoxin contamination in so far as the major compound present was aflatoxin G_1 . As far as we know no collaborative trial has been undertaken on samples which were so contaminated, all previous trials analysing material principally contaminated with aflatoxin B_1 . The samples, after preparation and before the trial took place, were analysed by BFMIRA using aqueous acetonitrile extraction, the Biocode clean-up procedure and HPLC (method based on reference 5). Mean results for the four contaminated samples were, respectively, 7.0, 9.2, 33.8 and 45.0 $\mu\text{g/kg}$ total aflatoxin. These results were similar to the results obtained for each respective sample in this collaborative trial and, as the majority of contamination in these samples was from aflatoxin G_1 , it can be suggested, even though this work with the Biocode column was carried out by only one laboratory, that the antibody present in the Aflaprep column has an equivalent affinity for aflatoxin G_1 than does the antibody used in the Biocode columns.

Conclusion:

For the analysis of aflatoxin contaminated peanut butter the method described in this report (using the Aflaprep column) will give analytical results of similar precision as the method described in the previous collaborative trials (using the Biocode column). This trial was undertaken by laboratories which undertake a wide spectrum of food analysis work and not all of which analyse aflatoxins regularly. If a further collaborative trial was organised on an international level with expert laboratories to test the Aflaprep method it may be anticipated that lower RSD_R values than those obtained here may be achieved.

Appendix I

Quantitative Determination by HPLC of Aflatoxins present in Peanut Butter using AFLAPREP Immunoaffinity Chromatography Sample Clean-up

1. Scope And Field Of Application

The method is designed to evaluate the Aflaprep immunoaffinity column for the determination of aflatoxins in peanut butter.

2. Definition

The content of aflatoxin: the concentrations of the aflatoxins B_1 , B_2 , G_1 and G_2 in peanut butter in $\mu\text{g/kg}$ as determined by the method.

3. Principle

The aflatoxins are extracted from the samples with a methanol-water mixture. A portion of the diluted extract is passed through the immunoaffinity column. The column is washed to remove non-specific interfering substances followed by

elution of aflatoxins from the affinity column. Individual and, hence, total aflatoxins can be quantitated by HPLC determination of the eluate.

4. Reagents

4.1 Sodium Chloride (Analytical Grade).

4.2 Methanol. Technical grade is suitable for the extraction solvent which consists of methanol : water (70:30 v/v). HPLC grade methanol is required for elution, preparation of the aflatoxin standards and the HPLC mobile phase.

4.3 Acetonitrile (Analytical Grade).

4.4 HPLC Mobile Phase. This solution consists of distilled water : acetonitrile : methanol (50:30:20 v/v/v).

4.5 HPLC Postcolumn Reagent (saturated iodine in water).

4.6 Aflatoxin Standards. Crystalline powder of aflatoxins B₁, B₂, G₁ and G₂ is resuspended in benzene : acetonitrile (98:2 v/v) to give a stock concentrate solution containing 1mg/ml. To prepare the aflatoxin standard working solution first dilute 50µl of the respective toxin stock concentrate to 5ml with methanol. Measure the absorbance of the toxin solutions in a U.V. spectrophotometer, set at 362nm, and blank against methanol.

Dilute each toxin solution with methanol to give 100ng/ml. Mix 1ml aliquots of the toxin solutions to give a mixture of 100ng total aflatoxins per 1ml methanol. To prepare the HPLC standard solution, dilute 1ml 100ng/ml total aflatoxins with 4ml acetonitrile : water (15:25 v/v). Inject 200µl standard solution into the HPLC to produce 4 peaks: each peak height or area is equivalent to 1ng toxin. The elution order is G₂, G₁, B₂, and B₁.

5. Apparatus

5.1 Laboratory Balance. Working range 1 - 100g, capable of accuracy to 1mg.

5.2 Blender. A high quality waring blender or equivalent incorporating a high speed motor, complete with 1 litre heat resisting glass container with lid and stainless steel cutter.

5.3 Filters. Whatman filter papers, 25cm, qualitative, grade 4. Whatman glass microfibre filters, 11cm.

5.4 U.V. Spectrophotometer.

5.5 Laboratory Glassware. Filter funnels; measuring cylinders for measuring 15ml, 30ml, and 125ml volumes; beakers (100-250ml capacity) for collecting sample filtrates and waste materials, 10ml amber vials for collecting eluates.

5.6 Clampstand.

5.7 Pipettes. Calibrated pipettes to cover the range 50-1000µl.

5.8 HPLC System. A typical system to be used was: 2 x Waters M501 pumps or equivalent to deliver (a) mobile phase at a flow rate of 0.8ml/min and (b) saturated iodine solution at a flow rate of 0.4ml/min. Waters U6K universal injector or equivalent to introduce 50-200µl sample volumes onto the column.

Hichrom guard column - Spherisorb ODS1, 5µm, 5cm x 5mm. Hichrom analytical column - Spherisorb ODS1, 5µm, 25cm x 5mm. Mixing T to introduce postcolumn reagent into column effluent and a reaction coil of 5ft. x 0.02 inch stainless steel tubing immersed in a water bath at 70°C. Waters 420 AC fluorescence detector or equivalent with 365nm excitation filter and 430nm emission filter. **5.9** Aflatest Kit Components. 25 immunoaffinity columns, 1 glass syringe barrel (10ml), 1 plastic syringe (pump unit), 1 rubber connector.

Table I
Aflatoxin level - ppb - Sample 1 (blank)

Laboratory No	Aflatoxin					
	B1		G1		Total Aflatoxin (B1,B2,G1,G2)	
1	0.00	0.51	0.00(a)	0.86(a)	0.00(a)	1.37(a)
2	0.00	0.00	0.00	0.00	0.00	0.00
3	0.45	0.52	7.00(b)	7.30(b)	7.45(b)	7.82(b)
4	0.50	0.50	0.50(a)	0.70(a)	1.40(b)	1.40(b)
5	0.00	0.00	0.00	0.00	0.00	0.00
6	0.40	0.20	0.00	0.00	0.40	0.20
7	0.00	0.00	0.00	0.00	0.00	0.00
8	0.60	0.60	0.00	0.00	0.60	0.60
9	0.00	0.00	0.00	0.00	0.00	0.00
10	0.10	0.40	0.00	0.00	0.10	0.40
11	0.00	0.60	0.00(a)	9.60(a)	0.00(a)	10.5(a)
12	0.00	0.00	0.00	0.00	0.00	0.00
13	0.40	0.00	0.00	0.00	0.40	0.00
14	0.20	0.30	0.20(b)	0.20(b)	0.40	0.50
15	0.30	0.20	0.00(a)	0.30(a)	0.50	0.60
16	0.00	0.00	0.00	0.00	0.00	0.00
17	1.8(a)	0.00(a)	0.00	0.00	1.80(a)	0.00(a)
18	0.00	0.00	0.00	0.00	0.00	0.00
19	0.40	0.40	0.00	0.00	0.40	0.40
20	0.00	0.00	0.00	0.00	0.00	0.00
21	0.00	0.00	0.00	0.00	0.00(a)	0.70(a)
Mean	0.19		0.00		0.19	
r						
SD _t						
RSD _t %						
R						
SD _R						
RSD _R %						

For Key, see Table VII

Table II
Aflatoxin level - Sample 2

Aflatoxin					
B1		G1		Total Aflatoxin (B1,B2,G1,G2)	
1.90	0.66	10.30	5.93	12.20	6.59
0.90	0.80	4.40	4.10	5.80	4.90
1.27	1.05	12.00	10.10	14.27	12.55
1.20	1.20	7.00	6.40	8.90	8.50
3.70(a)	0.00(a)	8.14	0.00	11.84	0.00
0.90	1.20	5.60	6.60	7.00	8.20
0.00	0.00	0.00	4.29	0.00	4.29
1.70	1.60	8.00	8.00	10.10	10.00
0.00	1.20	5.10	2.50	5.10	3.70
0.60	0.90	5.10	4.40	7.62	7.06
1.30	0.90	6.10	4.90	7.70	5.80
0.70	1.70	6.50	6.10	7.60	8.50
1.40	1.40	7.20	7.60	10.00	10.30
1.00	0.20	5.30	0.80	6.70	1.00
0.80	1.10	4.70	4.50	6.00	6.10
0.00	0.00	2.90	7.60	2.90	10.10
2.10	0.60	10.30	1.40	12.40	2.00
0.00	0.00	0.00	0.00	0.00	0.00
0.90	1.10	4.20	4.60	7.10	7.50
1.60	1.50	8.30	6.50	9.90	9.40
7.20(a)	12.50(a)	19.60(b)	22.20(b)	26.80(b)	38.70(b)
0.93	5.44		6.99		
1.24	6.90		8.80		
0.44	2.46		3.14		
47.45	45.32		44.96		
1.65	8.32		10.47		
0.59	2.97		3.74		
63.43	54.68		53.51		

6. Procedure

6.1 Sample preparation and extraction. Weigh 25g sample, 5g NaCl, and add 125ml methanol : water (70/30, v/v) into blender jar, cover and blend for 1 min at high speed. Immediately after mixing, pass 25-30ml sample extract through Whatman No. 4 filter paper. Measure 15ml filtrate and 30ml distilled water into a small beaker and mix. Refilter through glass microfibre filter immediately before passing through immunoaffinity column.

6.2 Unit Assembly. Aflatest immunoaffinity columns should be at ambient temperature prior to use. Ensure that the column has not dried out and contains buffer above the resin. Remove the cap from the top of the column, cut off the sealed end and replace on the column. Firmly attach the column to the 10ml glass syringe barrel and place in the clampstand. Ensure that the bottom plug of the affinity column is still in place.

Table III
Aflatoxin Level - Sample 3

Laboratory No	Aflatoxin					
	B1		G1		Total Aflatoxin (B1,B2,G1,G2)	
1	2.12	2.63	10.69	13.65	12.81	16.98
2	1.10	1.10	6.70	6.00	8.40	7.50
3	1.42	1.65	12.00	12.80	14.52	15.55
4	1.80	1.80	10.80	11.00	13.50	13.70
5	3.53(a)	0.00(a)	8.58	10.51	12.11	10.51
6	1.60	1.50	10.40	9.90	13.00	12.10
7	0.00	0.00	18.79(a)	6.24(a)	18.79(a)	12.51(a)
8	2.20	3.00	11.70	15.50	14.40	19.20
9	22.40(a)	0.00(a)	30.10(a)	7.80(a)	53.80(a)	7.80(a)
10	1.50	1.00	9.70	6.00	14.16	9.40
11	1.60	1.90	9.60	10.70	11.50	12.90
12	1.50	1.30	8.00	5.70	11.20	7.90
13	1.80	2.00	11.80	12.00	15.20	15.60
14	0.00	1.50	0.00	8.70	5.80	10.60
15	1.30	1.20	7.20	7.80	9.30	9.80
16	0.00	1.60	3.40	6.70	3.40	8.30
17	1.40	1.40	10.20	8.90	11.60	10.30
18	1.80	0.00	5.30	4.50	7.10	4.50
19	1.40	1.30	6.80	6.70	10.80	10.40
20	1.30	1.40	8.70	9.80	11.50	11.20
21	13.50(b)	9.20(b)	30.00(b)	29.60(b)	43.50(b)	38.80(b)
Mean	1.39		9.11		11.30	
r	1.44		3.82		5.47	
SD _t	0.51		1.37		1.95	
RSD _t %	36.83		14.99		17.29	
R	1.96		7.84		9.65	
SD _R	0.70		2.80		3.45	
RSD _R %	50.28		30.74		30.49	

Table IV
Aflatoxin Level - Sample 4

Aflatoxin					
B1		G1		Total Aflatoxin (B1,B2,G1,G2)	
8.54	7.50	52.34	30.26	62.27	38.86
4.20	4.10	22.20	21.50	29.10	27.80
2.32	2.02	17.20	15.10	23.22	20.72
5.80	6.00	36.00	36.50	43.70	45.30
4.13	3.53	22.42	20.72	26.55	24.25
4.80	4.70	29.70	29.30	37.60	37.00
0.00	2.83	7.25(a)	44.53(a)	7.63(a)	48.81(a)
8.90	9.60	45.50	47.50	56.20	59.10
2.50	1.60	12.20	14.60	15.90	16.20
2.40	6.70	11.90	19.00	18.38	27.62
5.40	5.20	31.40	31.20	38.00	37.60
4.90	4.10	21.00	17.50	28.20	24.40
5.70	5.40	34.90	35.70	46.20	46.60
4.50	4.30	26.50	24.80	31.90	29.90
7.40	4.00	37.00	21.70	47.30	28.30
4.00	1.70	33.00	14.00	39.00	18.30
4.00	3.60	24.50	19.00	28.50	22.60
3.60	3.40	19.90	19.60	25.20	23.00
3.80	4.70	20.20	22.10	28.10	32.10
5.20	4.00	33.40	27.20	41.60	33.90
19.80(b)	22.50(b)	67.80(b)	99.60(b)	87.60(b)	122.10(b)
4.53		26.28		33.17	
3.11		15.99		18.06	
1.11		5.71		6.45	
24.52		21.73		19.45	
5.67		27.56		32.92	
2.03		9.84		11.76	
44.73		37.46		35.44	

For Key, see Table VII

6.3 Immunoaffinity Chromatography. Remove the bottom plug from the immunoaffinity column and pass 15ml sample filtrate (equivalent to 1g original sample) slowly through the column using the hand pump. A flow rate of 2-3ml/min is strongly recommended. Collect the waste in a suitable container. Wash the column 2 times with 10ml distilled water. Ensure that all the residual water has been expelled from the column before the next stage. Place a 10ml amber collection vial directly beneath the column. Pipette 1ml methanol : acetonitrile (20:30 v/v) into the glass syringe barrel. Elute aflatoxins from the column by slowly passing the eluant through the column at a flow rate of 1 drop per second. Back flushing with the eluant 2-3 times is recommended to ensure complete denaturation of the monoclonal antibody with the subsequent release of aflatoxins into solution. Pipette 1ml distilled water into the glass syringe barrel, pass through the column and collect in the amber vial, to give a 2ml total volume.

Table V
Aflatoxin Level - Sample 5

Laboratory No	Aflatoxin				Total Aflatoxin (B1,B2,G1,G2)	
	B1		G1			
1	8.61	10.58	33.10	47.11	41.71	59.33
2	5.50	5.50	28.10	28.20	36.50	36.70
3	3.67	1.50	25.70	11.10	34.87	17.00
4	7.40	7.60	47.70	49.40	57.90	59.60
5	7.48	5.85	35.52	33.82	43.00	39.67
6	7.20	6.80	48.20	43.30	59.60	53.90
7	4.10	3.05	90.07	58.87	95.04	63.54
8	12.40	11.50	68.00	61.90	82.90	75.90
9	0.00	3.50	15.80	18.50	16.70	24.10
10	7.60	7.90	26.40	33.90	39.12	55.96
11	7.70	7.40	47.20	44.30	56.30	53.10
12	7.80	5.00	26.00	26.00	36.40	34.70
13	6.70	8.30	48.80	59.40	62.60	78.40
14	7.10	5.80	37.30	34.40	46.70	42.30
15	7.00	6.00	38.60	30.20	48.50	39.30
16	3.40	5.60	17.00	45.00	22.70	55.40
17	5.20	5.80	34.70	44.60	39.90	50.40
18	4.30	3.80	21.90	24.40	27.90	29.80
19	6.30	6.90	29.70	33.80	43.60	46.50
20	5.70	5.60	41.60	40.30	51.40	50.00
21	36.50(a)	28.70(a)	161.40(b)	155.90(b)	200.10(b)	185.80(b)
Mean	6.23		38.25		47.72	
r	2.96		22.66		26.62	
SD _t	1.06		8.09		9.51	
RSD _t %	16.97		21.16		19.92	
R	6.82		43.77		48.29	
SD _R	2.43		15.63		17.24	
RSD _R %	39.08		40.87		36.14	

For Key, see Table VII

Table VI

Collaborative Trial	Biocode trial U.K.		Biocode International Trial			Aflaprep - May & Baker U.K.Trial			
Aflatoxin level in sample ($\mu\text{g/kg}$)	12	35	4	15	38	7	11	33	48
RSD _R %	46	36	33	45	42	54	30	35	36

6.4 Quantitation of Aflatoxins by HPLC. Following start-up and equilibration of HPLC system, inject 200µl aflatoxin standard solution onto HPLC. The elution order is G₂, G₁, B₂ and B₁; each peak height or area is equivalent to 1ng toxin. Inject 200µl test sample (equivalent to 0.1g original sample) onto HPLC. Quantitate aflatoxin concentrations by comparing sample peak heights or areas to the standards.

7. Precautions

Aflatoxins are very hazardous substances. Rubber gloves, safety glasses and laboratory coats should be worn throughout the assay. All materials and reagents must be decontaminated by soaking for at least 2 hours in a solution of sodium hypochlorite (10% v/v), prior to rinsing thoroughly with distilled water. It is recommended that the glass syringe barrel is rinsed through with methanol and then distilled water at the end of each assay.

8. Expression Of Results

8.1 Calculation of aflatoxins: The concentration of aflatoxins, in µg/kg is given by:

$$(\text{AFLATOXIN}), (\mu\text{g/kg}) = \frac{\text{peak ht. or area of sample} \times 10}{\text{peak ht. or area of standard}}$$

Add the concentrations of the four aflatoxins to obtain total aflatoxin concentration.

8.2 Repeatability: To be assessed from the results of the collaborative trial.

8.3 Reproducibility: To be assessed from the results of the collaborative trial.

8.4 Relative standard deviation for reproducibility: To be assessed from the results of the collaborative trial.

TABLE VII

r	Repeatability (within-lab variation). The value below which the absolute difference between two single test results obtained under the same method on identical test material under the same conditions may be expected to lie with 95 % probability.
R	Reproducibility (between-lab variation). The value below which the absolute difference between two single test results obtained under the same method on identical test material under different conditions may be expected to lie with 95 % probability.
SD _r	The standard deviation of the repeatability.
SD _R	The standard deviation of the reproducibility
RSD _r	The relative standard deviation of the repeatability $SD_r \times 100 / \bar{x}$ ($\bar{x}$ = mean)
RSD _R	The relative standard deviation of the reproducibility $SD_R \times 100 / \bar{x}$ ($\bar{x}$ = mean)
(a)	Result rejected as outlier by Cochran's test ($p < 0.05$) values not used in calculation of mean, repeatability, reproducibility and relative standard deviation of repeatability and reproducibility
(b)	Result rejected as outlier by Grubb's test ($p < 0.05$) values not used in calculation of mean, repeatability, reproducibility and relative standard deviation of repeatability and reproducibility

Acknowledgements

The authors thank the following collaborators and their staff, for participating in the trial:

R.E.S. Andrews, Kent County Council, Maidstone;
R.J.C. Barron, Clayton Bostock Hill & Rigby, Birmingham;
H.M. Bee, County Analyst's Department, Stafford;
M.J. Billington, Birmingham Technical & Scientific Complex, Birmingham;
K.T. Chisnall, Derbyshire County Laboratory, Matlock;
E.C. Conchie, Ruddock & Sherratt, Manchester;
D. Dunn, A.H. Allen & Partners, Sheffield;
J. Duszanskyj, KP Foods, Leicestershire;
M.F. Godfrey, Lothian Regional Council, Edinburgh;
P. Hall, Dr. Bernard Dyer and Partners Ltd, London;
G. Keen, Hereford & Worcester County Laboratory, Worcester;
E.E.J. King, The Hampshire Scientific Service, Southsea;
D.W. Lord, Lancashire County Laboratory, Preston;
E.B. Reynolds, Tickle & Reynolds, Exeter;
D.G. Rixon, Mid Glamorgan County Public Health Laboratory, Cardiff;
B. Sanders, Cardiff City Analysts Laboratory;
T. D'Silva, Laboratory of the Government Chemist, London;
R.A. Stevens, Southwark Analytical and Scientific Services, London;
B. Taylor, Greater Manchester Scientific Services, Didsbury;
J.S. Turnbull, Avon County Scientific Services, Bristol;
J.P. Wootten, Central Scientific Laboratories, London.
Also thanked are G.M. Wood and P.J. Mann (BFMIRA) for analytical investigation and C. Clark (May & Baker Diagnostics Ltd, Glasgow) for donation of columns and protocol.

References

1. Association of Official Analytical Chemists (AOAC), 1990, Official Methods of Analysis of the Association of Analytical Chemists, edited by P.M. Scott, 15th edition (Washington D C : AOAC) Chapter 49.
2. Busby, W.F.Jr and Wogan G.N, 1984, Aflatoxins. Chemical Carcinogens, edited by C.E.Searle (Washington D C : American Chemical Society), 945 - 1136.
3. Mortimer, D.N., Sheperd, M.J., Gilbert, J. and Clark, C., 1988. ELISA determination of aflatoxin B₁ in peanut butter : collaborative trial. Food Additives and Contaminants, **5**, 601 - 608.
4. Newsome, W.H., J. Association of Official Analytical Chemists (AOAC). Potential and Advantages of Immunochemical Methods for Analysis of Foods. 919 - 922. Vol. **69**. No.6. 1986.
5. Patey A.L., Sharman M., Wood R., and Gilbert J..(1990) Determination of Aflatoxin Concentrations in Peanut Butter using an immunoaffinity column clean-up procedure: inter-laboratory study. Food Additives and contaminants **7**, 515 - 520.
6. Patey A.L., Sharman M., and Gilbert J..(1991). HPLC determination of Aflatoxin levels in Peanut Butter using an Immunoaffinity column clean-up procedure : International Collaborative Trial. JAOAC, **74**, 76-81
7. "Protocol for the Design, Conduct and Interpretation of Collaborative Studies" Ed W Horwitz, Pure and Appl Chem., 1988, **60(6)**, 855 - 864.
8. Van Egmond, H.P. (1989). Current situation on regulations for mycotoxins. Overview of tolerances and status of standard methods of sampling and analysis. Food Additives and Contaminants **6**, 139 - 188.